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A Mother Plant and Somatic Embryo Registry for the Propagation and Conservation of Artificial Seeds of the Orchid *Phragmipedium kovachii*

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Abstract: *Phragmipedium kovachii*, is an endemic orchid that naturally inhabits the humid zones of the Amazonas region in Peru. Currently, it has been extracted from its natural habitat, listed as critically endangered by CITES, suggesting searching for conservation strategies *in vitro* and in situ. Therefore, this research established an *in vitro* protocol to obtain and conserve artificial seed, through four events: (a) *in vitro* induction of the explant (sexual seeds from the dehiscent capsule), (b) Embryo germination, (c) Embryo development until the presence of plumule, (d) Encapsulation of the embryo in sodium alginate plus calcium chloride and then the conserving them under *in vitro* conditions. The Optimal morphology, floral formula, and floral diagram of the mother plant were characterized through digital image processing. The variables for the protocol were evaluated using the Kruskal Wallis ANOVA at the seed establishment stage. Treatments T1 (4.33 g DM +20 g sucrose +6 g agar +2 g activated charcoal; all at pH 5.2) and T2 (same conditions as T1 + 20% coconut water) of different culture mediums, were the most efficient in achieving seed germination in 43 days. For the days to plumule stage, T1 is the most efficient with 139 days. For encapsulation, an experimental trial was made using calcium chloride dihydrate ($CaCl_2$-$2H_2O$) at a concentration of 2% w/v and two doses of sodium alginate at 2 and 3% w/v. Both achieved encapsulation of the somatic embryo in the plumule stage at 219 days. All treatments were able to remain encapsulated for 365 days. Importantly, this research is a beginning for the preservation of endangered species with biotechnological techniques in artificial seeds.

Keywords: Seeds Production, Embryogenesis, *Phragmipedium peruvianum*

Introduction

Peru is recognized worldwide as a megadiverse country (Martel *et al*., 2021) with great biological richness and characterized by its diversity of climates, ecological zones, and production areas, which make it one of the most important centers of genetic resources (Oliva-Cruz *et al*., 2021) for plants and animals. However, the number of species at risk of extinction due to anthropogenic causes (Vohra and Gahlawat, 2020) is a critical indicator of the health of a country's biodiversity (Develey, 2021). Such is the case of the species associated with the Orchidaceae family and their conservation has become a global problem to be addressed in the scientific world (Mirenda, 2011).

Orchids comprise one-tenth of all systematically authenticated angiosperms and can thrive in different niches, from tropical basins to high alpine areas (Fatahi *et al*., 2022). These conditions make Orchidaceae one of the most diverse plant families on earth, representing 10% of the total plant biodiversity (Cuoco and Cronan, 2009). Having a high ornamental value (He *et al*., 2021) and commercial (Botelho *et al*., 2015; Hossain, 2015), orchids have aroused the interest of scientists, conservationists, and traders. Therefore, orchid species have been investigated because of their pollination,

reproduction, and small seed complex (Cozzolino and Widmer, 2005). Where the technique of *in vitro* multiplication of orchids is an alternative that can be used on a large scale to combat overexploitation of tubers and climate change (Fatahi *et al*., 2022).

On the other hand, *P. kovachii* was discovered by Faustino Medina Bautista, near his farm near Moyobamba and Chachapoyas, northern Peru; the name was published by Atwood *et al*. (2022a). By 2005, another study suggested that the species should be called *Phragmipedium peruvianum* (Craig, 2005). Given this case, legal action has been taken in Peru for the conservation of species and it first came to the fore when the "case of the stolen orchid" was denounced (Braem, 2004). Therefore, this species is included in Appendix 1 of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as a species in danger of extinction and whose trade control is quite strict (CITES, 2021).

In Peru, international trade in endangered species of flora and fauna is prohibited, only genetic material may be used for scientific research (MINAM, 2006). However, it is not only enough to control trade, it is also necessary to promote the conservation and management of wild orchid populations (MINAM, 2015). Therefore, within the conservation techniques, agricultural biotechnology plays a very important role, since it has a series of advances such as somatic embryos, which is a technique that allows the propagation and production of genotypes with superior germplasm (Fehér, 2019).

On the other hand, the combination of micropropagation and encapsulation techniques has allowed the development of a new tool, known as synthetic or artificial seed (Qahtan *et al*., 2019). Artificial seeds originate from somatic and non-zygotic embryos which open new perspectives in agriculture (Saiprasad, 2001). They are usually artificially encapsulated (Rihan *et al*., 2017). This improved technology is considered a technological alternative for the propagation of many important crops (Rihan *et al*., 2017) for economic, medicinal, and/or conservation purposes. The application of these artificial seeds makes it possible to maintain gene collections, which cannot be stored in liquid nitrogen (Faisal and Alatar Abdulrahman, 2019). Genetic constancy of the mother plant after germination of an artificial seed has been proven (Gantait *et al*., 2017a).

Official statistics suggest that most of the international orchid trade involves artificially propagated plants, with reported trade in wild-sourced plants accounting for <0.1% of the >1.1 billion live plants traded during 1996-2015 (Hinsley *et al*., 2015). However, a growing number of studies reveal that the volume of illegal and undeclared trade in wild-sourced plants far exceeds this legal component (Davenport and Ndangalasi, 2003; Phelps and Webb, 2015). Therefore, it is important to propose strategies that allow the use of technologies for the artificial multiplication of plants and the non-destruction of the habitat, as well as other factors that could affect the success of conservation. The unique contribution of this research is that for the first time, a strategy for *in vitro* multiplication and conservation of plant material of *P. kovachii* is proposed that will ultimately allow the care of the natural habitat and at the same time obtain economic gains.

Whereas the establishment of protocols for the production of artificial seeds could be convenient for the short-term storage and exchange of germplasm of a species between national and international laboratories (Gantait *et al*., 2017b), and seedling recruitment is essential for the sustainability of any plant population (Rasmussen *et al*., 2015). The present study aims to establish a protocol for *in vitro* propagation and artificial seed conservation of the orchid *Phragmipedium kovachii*, for which we performed (i) The characterization of the mother plant, (ii) The *in vitro* somatic embryo obtention of the identified species and finally (iii) The formation of artificial seeds by embryo encapsulation.

With this in mind, in this paper, we characterize the morphology of a mother plant with high-resolution digital images, including for the first time flower bud, mature and dehiscent capsule, sexual seed morphology captured from a stereoscope, as well as floral formula, and floral diagram. Besides, a protocol for obtaining and preserving *in vitro* artificial seeds of this orchid species is included. Different treatments were evaluated at each stage of the process of obtaining artificial seed. At the seed establishment stage, the treatments with different culture methods, T1 and T2, were the most efficient, achieving seed germination in 43 days. For days to the plumule stage, somatic embryos were obtained from seeds in *in vitro* culture medium, T1 being the most efficient with 139 days. For encapsulation, we applied calcium chloride dihydrate ($CaCl_2$-$2H_2O$) at a concentration of 2% w/v and two doses of sodium alginate at 2 and 3% w/v. An *in vitro* protocol is presented to obtain an artificial seed of *P. kovachii*, which was able to be preserved under optimal conditions for no less than 365 days.

Materials and Methods

Morphological Characterisation of the Mother Plant of Phragmipedium kovachii

The mother plant of *P. kovachii* was obtained from the KGORY THIKA nursery located in the Shucayacu annex, Yambrasbamba district, Bongará province, Amazonas department, Peru. It is in charge of Mr Jhon Charles Valle Mas; who has authorization N° 004-2015-GRA-ARA-DEGBFS-A, BONG/PCFFS-POM/LARCH, to run the nursery for commercial purposes. The research permits for the species were obtained from SERFOR with general

directorate resolution N° D000109-2021-MIDAGRI-SERFOR-DGGSPFFS.

The plant material was taken to the Laboratory of Dendrology and Herbarium KUELAP, of the National Scientific Institution Depository of Biological Material (ICNDMB) with authorization code N° AUT-ICND-2020-001, belonging to the National University Toribio Rodríguez de Mendoza de Amazonas (UNTRM); where the confirmation of botanical determination of the species and its registration in the herbarium was carried out.

The morphological characterization of the mother plant was followed by digital image processing suggested by Haimovich and collaborators (David *et al*., 2012). Digital images were collected every day for 8 days. Each photograph shows the image of the plant with position detection of each of its parts. Then, measurements were taken for width, length, and height, as well as digital images of the two parts. The shape, size, and color of each plant part were recorded against Atwood (as *P. kovachii*) and Christenson (as *P. periuvianum*) (Atwood *et al*., 2002b; Cribb, 2005). In this research, we included information about the characterization of the flower bud, mature and dehiscent capsule, and sexual seed morphology captured from a stereoscope, in the mother plant. The floral formula and floral diagram were recorded for *P. kovachii* using the recommendations of Prenner (Prenner *et al*., 2010).

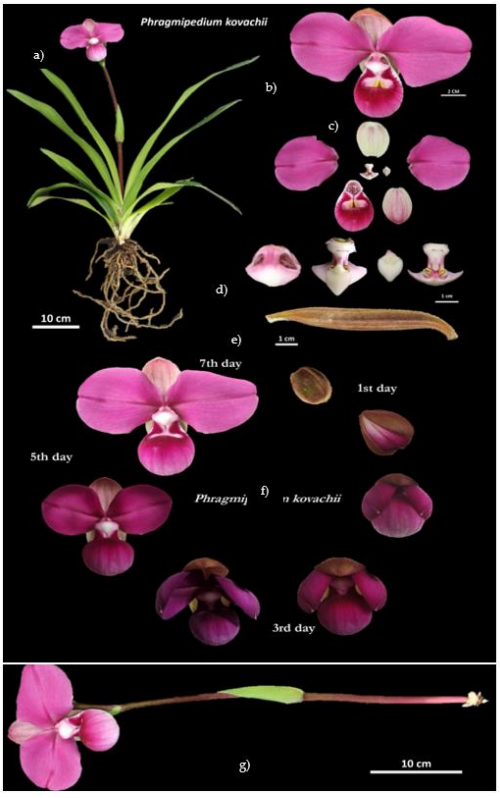

Fig. 1: *Phragmipedium kovachii*; (a) Whole plant; (b) Petal; (c) sepal; (d) Flower column; (e) Capsule; (f) Flower opening process; (g) Stem with flower

Collection of Plant Material and in vitro Propagation by Somatic Embryogenesis of Phragmipedium kovachii

A fully mature capsule was used, before dehiscence (Figs. 2-4). When collected, it was refrigerated until the time of *in vitro* sowing in the Laboratory of Plant Physiology and Biotechnology of the UNTRM. Five different treatments were prepared (Table 1), corresponding to culture medium with different concentrations tested on other orchid species (Billard *et al*., 2015; Lee-Espinosa *et al*., 2009; 2010; Segretín, 1992; Pérez Martínez, 2015).

Murashige and Skoog, sucrose, agar, and slightly acidic pH regulated with Sodium Hydroxide (NaOH) and Hydrochloric Acid (HCL) were used as the culture medium base for all treatments. Buffer substances such as activated charcoal (at T1, T2, T5); coconut water (at T2, T4); banana flour (for T4, T5); myoinositol, NAA, BAP and IAA (at T3) were added to the treatments, in the order listed (Table 1). Sterile distilled water was used for dilution. Each treatment was placed in 10 flasks, with 25 mL of medium in each.

The prepared culture medium was poured into a glass magenta with a Snap-on lid, autoclaved with a sterilizations time of 20 min at 120°C, and then refrigerated until seeding. Using 70% alcohol, the cabinet, materials, instruments, and culture medium were disinfected (Tejada-Alvarado *et al*., 2023). The plant material (capsule before dehiscence containing the sexual seeds) was disinfected with liquid soap for 5 min, rinsed 3 times, and placed in 4% sodium hypochlorite to enter the laminar flow cabinet without any external agent. The laminar flow cabinet was sterilized with UV rays for 15 min. After the air was turned on, the capsule was immersed in 95°C alcohol and flamed, this procedure was done twice. The capsule was placed in a sterile petri dish and the capsule was cut longitudinally with a No. 11 scalpel. The uncovered seeds were placed in magentas containing the culture medium, covered with a layer of sterile distilled water, closed, sealed with parafilm, and labeled. The magentas were taken to a growth environment and evaluated days to germination, with a photoperiod of 18 h light and 6 h dark, illuminated with artificial light, at a room temperature of 22°C, regulated by air conditioning.

Evaluations were regularly carried out every week. The germinated embryo condition was when the seed managed to break the seed coat, swell, and show a greenish coloring (Fig. 5A-B). Days to the plumule stage were counted until the time of the appearance of the structure (plumule) (Fig. 5C-D).

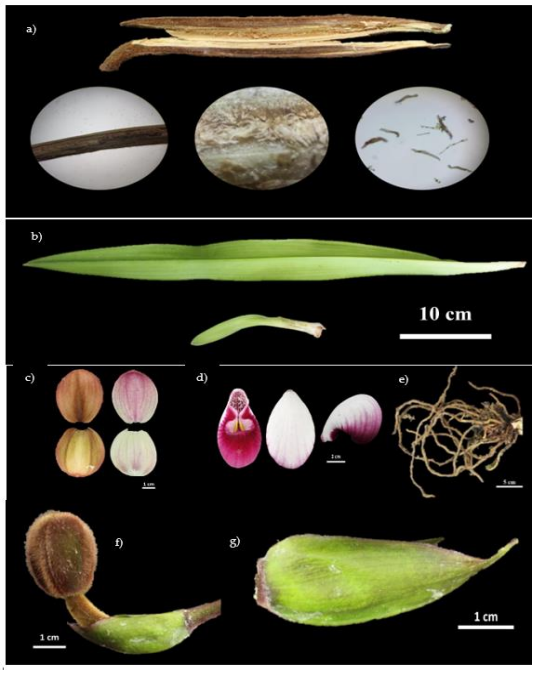

Fig. 2: *Phragmipedium kovachii*; (a) Morphology of the seed taken with a stereoscope); (b) Leaf; (c) Sepal; (d) labellum, (e) Root; (f) Floral bud; (g) Floral bract

Fig. 3: *Phragmipedium kovachii*; (a) Ovarian morphology; (b) Mature capsule; (c) Mature dehiscent capsule

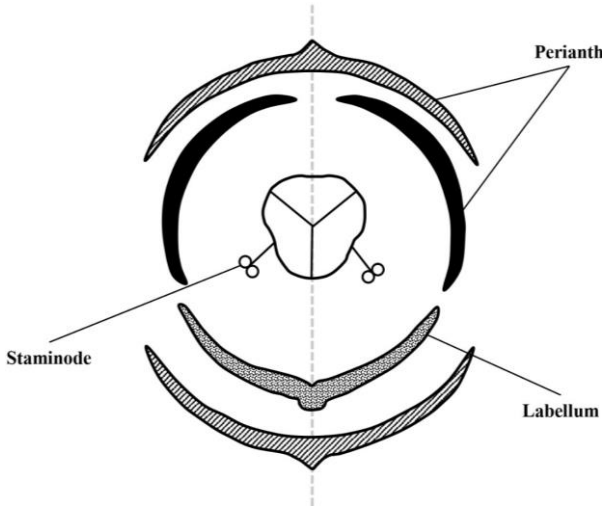

Fig. 4: Floral diagram of *P. kovachii*

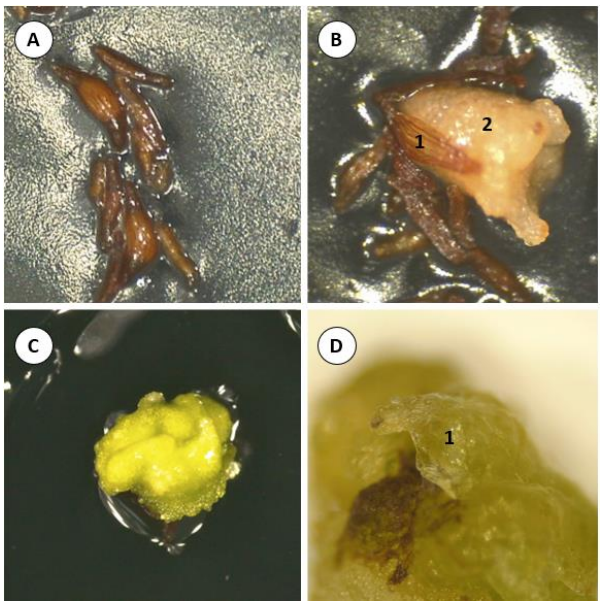

Fig. 5: Obtaining somatic embryos in plumule stage of *P. kovachii*; (A) Ungerminated seed; (B) 1. Remains of seed envelope, 2. Germinated embryo; (C) Germinated embryo; (D) 1. Embryo in plumule stage

Using Kruskal-Wallis ANOVA ($p<0.05$) (Cavalcante *et al*., 2012; Scriven *et al*., 2013), the effect of culture medium on the variables: Days to germination, days to plumule stage, and embryo at plumule stage was evaluated. Multiple comparisons were carried out to compare the means of the results of each variable concerning the effect of the treatments using Duncan's test (Feng and Chen, 2014; Nongdam and Tikendra, 2014; T-Thienprasert *et al*., 2021).

Table 1: Culture media established for different treatments for propagación *in vitro* de *Phragmipedium kovachii* by somatic embryo obtention

	T1	T2	T3	T4	T5
MS	4.33 gr/L	4.33 gr/L	4.33 gr/L	4.33 gr/L	4.33 gr/L
Agar	6 gr/L	6 gr/L	6 gr/L	6 gr/L	6 gr/L
Sucrose	20 gr/L	20 gr/L	30 gr/L	30 gr/L	30 gr/L
Activated carbón	2 gr/L	2 gr/L	--	--	2 gr/L
Banana flour	--	--	--	30 gr/L	40 gr/L
Coconut wáter	--	20%	--	0.5%	--
Myo inositol	--	--	100 mg/L	--	--
1- Naphthaleneacetic Acid (NAA)	--	--	2 mg/L	--	--
Benzylaminopurine (BAP)	--	--	2 mg/L	--	--
indole-3-acetic acid (IAA)	--	--	2 mg/L	--	--
pH	5.2+0.2	5.2+0.2	5.7+0.2	5.2+0.2	5.2+0.2

Forming Artificial Seeds by Embryo Encapsulation

Artificial seeds were obtained by encapsulation of the somatic embryos after plumule emergence. Two culture mediums were prepared, the first solution is calcium chloride dihydrate ($CaCl_2$-$2H_2O$) at a concentration of 2% (W/V), adding MS at 100%, with pH regulated at 5.7±0. 2; the second one is Sodium Alginate at two concentrations of 2 and 3% (each dose corresponding in evaluations as two treatments, where T1 = 2% and T2 = 3%), adding MS at 100%, regulated at a pH of 5.7±2; both culture mediums were autoclaved at 121°C for 20 min. For each treatment, 50 artificial seeds were developed.

Disinfection of the laminar flow cabinet, materials, and instruments was done with 70% alcohol and UV rays for 15 min (Mállap-Detquizán *et al*., 2022). The somatic embryos were removed from the flasks with culture medium and poured into the flasks containing sodium alginate. Then, with a dropper, the embryo was sucked together with the sodium alginate and poured as a drop containing one embryo, into the solution containing calcium chloride dihydrate according to each treatment. It was stirred for 30 min to shape the capsule; the calcium chloride medium was then decanted and the capsules were rinsed three times with sterile distilled water. They were placed in a sterile petri dish with a layer of water to prevent dehydration. The petri dish was sealed with parafilm. It was labeled with the treatment number and date and placed in the acclimatization environment at a temperature of 22°C with artificial light at a photoperiod of 16 h light and 8 h dark.

Protocol for Production and Preservation of Artificial Seeds

The identification of a protocol was based on the recording of four events: (a) *in vitro* induction of the explant (sexual seeds from the dehiscent capsule), (b) Embryo germination, (c) Embryo development until the presence of plumule, (d) Encapsulation of the embryo in sodium alginate plus calcium chloride. The protocol was developed by choosing the best treatments meeting the variables: Days to germination, days to plumule stage, and characteristics of seed encapsulation.

Results

Morphological Characterisation of the Mother Plant of Phragmipedium kovachii

Phragmipedium kovachii was collected in a monopodial growth habit. The root is of the lithophytic growth type (does not have a velamen). The root average size obtained is 29.2 cm (maximum of 49.5 cm and a minimum of 7.5 cm) and an average number of roots of 19, with a light brown color. The stem is cylindrical and the measurements obtained from the plant sample are 40.8 cm long and 0.9 cm thick, with a light pink coloring at the base of the leaves and a green color at the top, with reddish trichomes that give it a reddish-brown color.

The oldest leaves are larger with a length of 53 cm and a width of 5 cm and the youngest is smaller with a length of 15 cm and a width of 1.8 cm. Also, the leaf tonality varies according to the age of the leaf, the older ones are darker than the young ones and the upper side of the leaves are darker than the underside.

The floral bract of this plant sample has a length of 3.6 cm per 1.3 cm wide and is funnel-shaped with a tip at the top, with a light green color. An immature ovary was characterized, measuring 7 cm long and 0.8 cm wide, and has a generalized light brown color with reddish trichomes. The flower is zygomorphic, 14.5 cm long and 11.8 cm wide, consisting of 2 sepals, 2 petals, 1 labellum, and 1 column. The sepal covers and protects the flower parts up to the opening of the flower.

The flower bud when closed, developed the flower is divided into 2 for the expansion of the flower and is located at the back. It is 4.8 cm long and 3.7 cm wide. The characteristic of the ends being slightly curved inwards, the external part that was more exposed to the environment is green with brown and reddish sectorial tonalities with red trichomes; while the internal part presents a whitish tonality with violet tonalities in line, with a smooth texture.

Table 2: Symbol and meaning of the floral formula of the species *P. kovachii*

Symbols	Meaning
♂	Hermaphrodite or bisexual
⬇	Zigomorpha
P	Perianth
A	Androecium
$\bar{G}$	Gynoecium ∞ many 3 ovarian cavities
+	Double whorls
,	Separate each whorl
( )	Parts of the whorl are joined

Table 3: Effect of 5 culture medium on embryo germination and plumule generation for *p. kovachii*

Variable	T1	T2	T3	T4	T5
Days to embryo germination **	43+0,9^{b}	43+1,9^{b}	0^{a}	0^{a}	56+1,8^{c}
Days to plumule state **	139+1,6^{b}	160+2,7^{c}	0^{a}	0^{a}	185+2,7^{d}

**Statistical significance

The flower has 2 large showy petals. The measurements of the plant material obtained are 7.2 cm long and 6.6 cm wide, they are slightly curved outwards, the front part has a lighter purple color, and the back part a darker purple with a part that fades to a white tone towards the central internal part, it also has the characteristic of having a horizontal line that crosses the petals with a color differentiated by the tone.

The collected labellum measures 7.6 cm long, 5.5 cm wide, and 4.2 cm high. Morphologically, it is sac-shaped, white at the back, changing color towards the front with a purple color. It becomes darker purple at the apex, before entering the interior of the labellum it turns pale pink with two yellow stripes. Inside the labellum, white trichomes can be observed with a white background and purple dots.

The column of *Phragmipedium kovachii* is the central reproductive part of the flower. It consists of two dull yellow anthers with a close stigma for better efficiency in pollen reception, the column is 2.6 cm wide and 2.3 cm long with the recurved apex inwards and external and internal colors varying from pale pink to white in different sectors.

It has a round morphology, with a 2.7 cm by 2.1 cm size, with a dark green color and brown to reddish trichomes. *Phragmipedium kovachii* has an elongated, circular capsule, which forms behind the flower. It is 8.6 cm long and 1.1 cm wide. It is light brown, with some green tones in places, and also has reddish trichomes. It also has a capsule in a state of dehiscence, which opens naturally so that the pollen is transported by the wind, as the seeds are very small and are released as dust. The plant material collected was not yet in mature form. The seeds are attached to their base, where we can see the dark embryo microscopically, which is covered by a testa of dead cells.

The flower formula of *Phragmipedium kovachii* was identified as follows:

$$♂ ⬇ P2 + 3, [A\ (2)\ \bar{G}\ (1) \begin{bmatrix} \infty \\ 3 \end{bmatrix}$$

The species *Phragmipedium kovachii* is a hermaphrodite plant; with a zygomorphic flower; the union of its 2 sepals, 2 petals, and 1 labellum are called perianth with a double whorl; in the following whorl we have by union the androecium which has two anthers and the gynoecium with an inferior ovary, with 3 ovarian cavities and a finite number of ovules. For the floral formula of *P. kovachi*, a total of 8 symbols and the meaning of each is presented in Table 2,

Floral Diagram

The graphic representation of the arrangement of the floral parts of the species *Phragmipedium kovachii* shows that the flower is of double symmetry, with the presence of staminodes, labellum, and perianth, which is the union of the bracteole and petals; shown in the diagram (Fig. 4).

Somatic Embryo Collection in the Plumule State

The effect of 5 growing mediums on germination was evaluated (Table 3). Significant statistical differences were identified for the treatments ($p<0.05$). The highest value was found for treatment 5 with 56+1.8 days to embryo germination. Whereas, treatments 3 and 4, only reached germination at the final date of the experiment at 223 days. The effect of the culture medium in treatments 1 and 2 allowed embryo germination to be achieved in a shorter number of days (43 in both cases).

The variable days to the plumule stage showed significant statistical differences for the treatments. The highest value was found for treatment 5 with 185+2.7 days to the plumule stage of the embryo. Treatments 3 and 4 only reached the plumule stage at the end of the experiment at 223 days. With T2, the plumule stage was reached at 160+2.7 days. Meanwhile, the effect of the culture medium in T5 allowed the plumule stage to be obtained in a shorter number of days compared to the other treatments (139+1.6).

Forming Artificial Seeds by Embryo Encapsulation

An artificial seed was obtained by encapsulation of somatic embryos in the plumule stage with both doses, treatment 1 with 2% sodium alginate and treatment 2 with 3% sodium alginate. The artificial seed was preserved for at least 365 days in both cases. However, with 2% sodium alginate, we obtained a deformed structure, with some embryos protruding from the capsule and prone to external agents interfering with embryo development (Fig. 6A-B). In contrast, with 3% sodium alginate, the circular capsule shape was more uniform, and the embryo was centered and protected by the outline of an external agent (Fig. 6C-D).

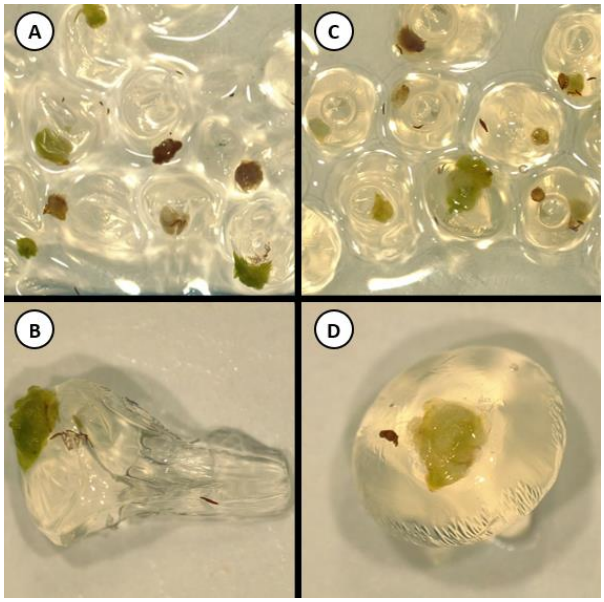

Fig. 6: *P. kovachii* artificial seed with embryo in plumule stage, T1 (A and B) and T2 (C and D)

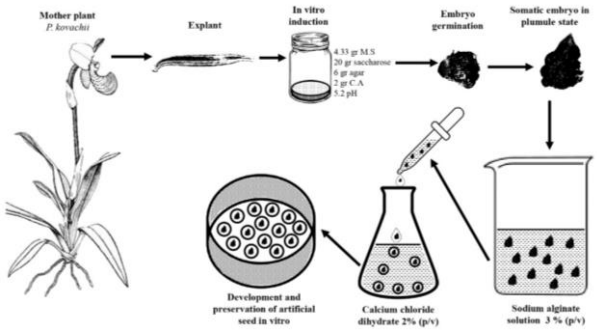

Fig. 7: Protocol for the development and production of artificial seed for *in vitro* conservation of *P. kovachii*

Protocol for the Production and in vitro Preservation of Artificial Seeds

Figure 7, presents a protocol for the production and *in vitro* preservation of artificial seeds of *P. kovachii* from germinated somatic embryos in the plumule stage. We used the results of the previous objectives where embryo germination and plumule stage were achieved in a shorter number of days. Also, the treatment of best behavior and capsule formation was used in the process of obtaining artificial seed.

Seeds from a fully mature capsule, before dehiscence, obtained from a mother plant were used. *In vitro*, induction was performed with the culture medium containing: 4.33 g/L DM, 6 g/L agar, 20 g/L sucrose, and 2 g/L activated charcoal, at pH 5.2+0.2. The embryo should germinate at 43 days and at 139 days the plumule emergence should be achieved. Encapsulation is then done with 3% sodium alginate followed by the application of 2% calcium dihydrate. Finally, the artificial seed will be preserved *in vitro* for at least 365 days.

Discussion

Research done by Atwood *et al*. (2002b), described some general characteristics of *Phragmipedium kovachii*, regarding the size of roots, leaves, inflorescences, flowers, bract, ovary, sepal, staminodes, and petals. In 2003, Koopowitz graphically described its habitat based on a visit to the only super-living population, on unshaded limestone cliffs (Craig, 2005). The location details that it was growing to the southwest, at an altitude of about 85 m, in the primary montane forest from 1900-2000 m a.s.l. Additionally, this research shows graphically and thoroughly each of the parts of a mother plant, emphasizing characteristics described for the first time with respect to the unripe flower bud (flower bract, ovary) and floral formula.

There are two types of roots in orchids, determined by growth habit. Roots with velamen function to capture water and absorb nutrients and those without velamen serve to support the plant (MINAM, 2015). Epiphytes have velamen and lithophytes and terrestrials have no velamen (Menchaca García, 2011), so *Phragmipedium kovachii* is of lithophyte growth habit, having no velamen (Fig. 2). Generally, in orchids, they are classified into three distinct stem types. The cylindrical stem, pseudobulbs, and corms (Menchaca García, 2011). Cylindrical stems are elongated and erect, with internodes where leaves and inflorescence emerge, typical in *Phragmipedium kovachii* (Fig. 3).

In all orchids, the leaves are simple with no relevant features such as columns, serrated or other, usually elongated and narrow, which can be preserved for many years (Menchaca García, 2011). There are 3 types of leaves in orchids; folded, conduplicate, and cylindrical (Ambiente, 2015; 2017). *Phragmipedium kovachii* has the folded leaf type. Cribb (2005), described *P. kovachii* as leathery, linear-lanceolate, acute or acuminate leaf type, up to 55 cm long, 5 cm wide, bright green or yellow-green above and with a white color texture, as shown in Fig. 4, in this study.

Regarding orchid flowers, they all have a basic floral pattern (Díaz-Toribio, 2013), with three distant sepals; and three petals, two of them dorsal and equal to each other and different from the third, which is called labellum (William, 2013). It acquires the oddest shapes for pollination efficiency and the reproductive organs are fused into a single structure called a column (Díaz-Toribio, 2013). *Phragmipedium kovachii* has a purple flower color and they are unique in the genus, which specializes in green, yellow, and brown flowers (Cribb, 2005). The

flower of the mother plant used in this research reached 14.5 cm from petal to petal at 5 days from the onset of flowering. A *P. kovachii* was illustrated with the largest flowers seen so far, with a length of almost 20 cm from petal tip to petal tip (Cribb, 2005), presumably the time at the end of flowering.

The ovary is defined as the organ of the flower that contains the ovules and in the orchids it is found below the other parts of the flower (William, 2013). But, in *P. kovachii*, it is below the other flower parts, with trilocular, and stores a large number of ovules to become seeds at maturity state (Ajú Upún, 2009). *P. kovachii* has a labellum, which has a larger and showier characteristic than the other parts of the flower, which is often attached (Rivera and Honduras, 2002). This part of the flower acts as an attractant for pollinating insects, attracting them with special shapes and eye-catching colors (De Investigaciones and Extensión, 2015). The labellum morphology defines the different orchid genera (Ajú Upún, 2009).

In orchids, the sexual organs of the flower are attached to a column, which holds the anthers and the stigma (De Investigaciones and Extensión, 2015). *P. kovachii* has a short column; convex staminode, transversely elliptic and glabrous (Cribb, 2005), as shown in Fig. 1. It also has a bract, referring to a leaf organ, which has the function of protecting the reproductive structures (Ambiente, 2017), as shown in Fig. 2. The anther is the source of several of the main characters traditionally used for classification in Orchidaceae (Freudenstein *et al*., 2002; Singer, 2009). *P kovachii* has two dull yellow anthers, a distinct characteristic from 99% of orchids, which have a single anther (*Vanilloideae*, *Orchidoideae*, *Epidendroideae*) (Freudenstein *et al*., 2002). Orchid pollen is agglomerated, forming a mass called pollinium, which has a flared, sticky end, which serves for the pollinium to adhere to the pollinating insect (Ajú Upún, 2009). The intrusive/juxtaposed orientation of the pollinium in *Phragmipedium* (Vermeulen, 1966), arises by the reorientation of the thecae so that the stomata become adaxial (Freudenstein and Rasmussen, 1996). Intrusive anthers characterize all orchids except those with overlapping pollinia (Freudenstein *et al*., 2002).

The fruits of orchids are botanically denominated capsules (Menchaca García, 2011). Inside each capsule are seeds of very small size (Ambiente, 2015). In *P. kovachii*, these capsules are about 8-10.5 cm in length and 0.8-0.9 cm in diameter (Cribb, 2005), illustrated in Fig. 3. An orchid capsule can comprise up to two million seeds, which usually lack endosperm and have an undifferentiated embryo with a single cotyledon (Lee-Espinosa *et al*., 2009). The seed is wind-spread, living in symbiosis with a fungus for germination and initial development (Ajú Upún, 2009).

In this research, we successfully photographed the mature dehiscent capsule of *P. kovachii* in an artificial habitat on rocks. Of the three growth types recorded in orchids: Lithophytic, epiphytic, and terrestrial (Ambiente, 2015), *P. kovachii* belongs to the lithophytic orchids (Fig. 3). This orchid, often in large compact clusters, forms short and stout rhizomes (Cribb, 2005). The main characteristic is that it grows on rocks, feeding on rock mosses, dissolved nutrients from rainfall, rock debris, and dead rock tissue (Ajú Upún, 2009).

Since Morel first cultivated Cymbidium shoot meristems, modern biotechnology has reshaped orchid research and revolutionized the orchid industry (Arditti and Krikorian, 1996). Studies are also needed to find a way to obtain *in vitro* multiplication to continue the life cycle of this orchid species and to maintain and recover its wild populations (Li *et al*., 2008), as well as *in vitro* conservation of ornamental plants (Bonilla Morales, 2015). Using a mother plant for *in vitro* multiplication is of vital importance and it must retain characteristics of varietal purity and be free of pests and diseases (FAO, 2012). Closed capsule seed was used to avoid contamination before dehiscence, which starts with inoculation of the seed into the culture medium for seed germination (Lee-Espinosa *et al*., 2010).

Authors use a solidified base medium for the *in vitro* establishment of orchid seeds before dehiscence or in capsule, which is set by Murashige and Skoog, with sucrose, agar, and slightly acidic pH (Billard *et al*., 2015; Segretín, 1992; Pérez Martínez, 2015). Some authors add substances that allow better germination efficiency, such as activated charcoal (Vaca *et al*., 2018), which was used at T1, T2, and T5; coconut water (Lee-Espinosa *et al*., 2009) (Utami and Hariyanto, 2019), which was used at T2 and T4; banana flour (Pérez Martínez, 2015), which was used at T4 and T5; Myo inositol, NAA, BAP and IAA (Lee-Espinosa *et al*., 2010), which was used at T3, data given in Table 1.

For encapsulation, plumule stage embryos, without roots, were selected 43 days after *in vitro* development (Fig. 5). Treatment 1 achieved the plumule stage at 139 days. *Laelia anceps* species achieved the plumule stage approximately 3 months after *in vitro* seeding using embryogenic callus embryos grown from mature zygotic embryos 40 days after *in vitro* development (Lee-Espinosa *et al*., 2009). The difference in time is not only due to the genetic characteristics of the plant but also to environmental conditions, temperature, and other factors (Cortes *et al*., 2017). Advances in somatic embryogenesis have been made for several species in recent years (Lee-Espinosa *et al*., 2009), however, no new synthetic seed coatings have been developed since the early 1980s when polyoxyethylene and sodium alginate were first used. Treatments 1, 2, and 5 of this research, successfully developed somatic embryos, defined by the presence of the plumule stage.

Coconut water (T2) and banana flour (T5) were present in treatments that took a greater number of days to obtain somatic embryos. MS culture medium (like T3 of this investigation), supplemented with 2 mg-litre-1 of NAA, BAP, and IAA, was used for *Laelia anceps* ssp. dawsonii. Three-month embryos were obtained from *in vitro* seeding (Lee-Espinosa *et al*., 2009). The non-development of embryos at T3 in this research demonstrates the importance of the genetics of the species and of supplements to be added to the medium (such as growth hormones) in the performance of a protocol (Pérez Martínez, 2015).

Germination of somatic embryos in orchids has been reported to be obtained in record days. *Brassia verrucosa*, for example, germinated after 6 days (using as medium components: M and S, Sucrose 30 g/L, Coconut water 100 ml/L, Apple, banana and tomato 40 g/L, activated charcoal 1 g/L, Agar 6.5 g/L); at 13 days, *Oncidium stramineum* embryos were obtained (with M and S, sucrose 30 g/L, agar 6.5 g/L, pH 5.7) (Flores-Escobar *et al*., 2008). However, the timing of the embryo procurement process depends on the age, the appropriate developmental stage of the explant, the explant development medium, the effective medium, and other appropriate physical and chemical conditions (Attree and Fowke, 1995).

It can be stated that synthetic seed technology is not very old (Levitus *et al*., 2010), the oldest data is from the 1970s when Professor Murashigue mentioned to his students at the University of California that "someday somatic embryos may be encapsulated, thus becoming artificial seeds" (Orlando *et al*., 2004).

Countries in which this technique is highly developed include Japan, China, the United States, France, Spain, Canada, and developing countries (Orlando *et al*., 2004). The artificial seed can regenerate a plant identical to its parent (Lee-Espinosa *et al*., 2009). The artificial seed technique allows the storage of seeds, meristems, apices, pollen, callus, and cell suspensions and is considered a useful tool to avoid somaclonal variations and to allow the conservation of explants for indefinite storage time (Bonilla Morales, 2015). The technology to produce synthetic seeds allows plants with low germination percentages to be obtained in short periods (Bonilla Morales, 2015) Currently, the germplasm of many plant species is being lost, so the advance of artificial seed production represents a conservation alternative (Lee-Espinosa *et al*., 2010).

Artificial seed facilitates the handling, transport, and preservation of plant species, contributing to the rescue of biodiversity; it protects the embryo from mechanical damage, they must be soft enough to break the cover when germinating and the hydrogels used must have the capacity to retain nutrients to feed the embryo (Bonilla Morales, 2015). Early research on artificial seeds used polyoxyethylene for encapsulation, then gelling agents such as alginate, carrageenan, guar gum, and sodium pectate were investigated, but alginate was found to be optimal for developing this technique, due to its sensitive thickness, low toxicity to microorganisms, low cost and rapid gelling (Rihan *et al*., 2017).

Currently, sodium alginate is one of the most widely used polymers for artificial seed encapsulation, as it has a suitable hardness (Bonilla Morales, 2015). Capsule formation depends on ion exchanges between sodium alginate and calcium chloride, where it occurs when a drop of sodium alginate is dropped with the embryo into a solution of calcium chloride dihydrate, the solidity and rigidity of the capsule depends on the gelling agents (Rihan *et al*., 2017).

Remarkably, in the research done by Chandrasekhara (2012), he reported studies carried out on artificial seed, finding 276 studies worldwide up to that time. The research reported in this section strongly encourages the future use of our research to preserve *in vitro* artificial seeds of *P. kovachii* and further studies are encouraged, based on this one, to know the viability of the seeds during prolonged periods to evaluate the efficacy of the conservation technique. Since this research, we included for the first time the flower bud, mature and dehiscent capsule, and sexual seed morphology captured from a stereoscope.

Conclusion

The morphological characterization of the species *Phragmipedium kovachii* was carried out, according to the morphological characteristics mentioned by Atwood *et al*., (2002a). Artificial seeds of the species *Phragmipedium kovachii* were established in an *in vitro* medium, mediante four events: (a) *in vitro* induction of the explant (sexual seeds from the dehiscent capsule), (b) Embryo germination, (c) Embryo development until the presence of plumule, (d) Encapsulation of the embryo in sodium alginate plus calcium chloride and then conserving them under *in vitro* conditions. The somatic embryos with the most efficient treatment at 43 days and reached the plumule stage at 139 days in the most efficient treatment. The artificial seed of *Phragmipedium kovachii* was preserved by encapsulation of the somatic embryo in the plumule stage 3% sodium alginate for encapsulation being the most resistant to dehydration; the encapsulated embryo was preserved for no less than 365 days.

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Author's Contributions

Osber Cunia: Mad a major contribution to the conduct of the research and data acquisition.

Manuel Oliva: Coordinated the data analysis and contributed to the writing of the manuscript.

Segundo G. Chavez: Contributed to drafting the article or reviewed it critically for significant intellectual content.

Jaris Veneros and Grobert A. Guadalupe: Critically reviewed and provided substantial edits to the manuscript.

Manuel Ix: He granted final approval to the version to be presented and to the revised version.

Ligia Magali García Rosero: Create concept and design, monitor the progress of research from start to finish, and ensure that the entire research process goes according to plan, analysis, and interpretation of data.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

Data Availability

The data used to support the study are available upon request.

References

Ajú Upún, M. M. (2009). *Las orquídeas bases generales para su conocimiento y enseñanza*. Universidad de San Carlos de Guatemala.

Ambiente, M. del. (2015). *Guía de identificación de orquídeas con mayor demanda comercial*.

Ambiente, M. del. (2017). *Orquídeas del Perú y herramientas para su identificación*. MINAM.

Arditti, J., & Krikorian, A. D. (1996). Orchid micropropagation: the path from laboratory to commercialization and an account of several unappreciated investigators. *Botanical Journal of the Linnean Society*, *122*(3), 183–241. https://doi.org/10.1111/j.1095-8339.1996.tb02073.x

Attree, S. M., & Fowke, L. C. (1995). Conifer Somatic Embryogenesis, Embryo Development, Maturation Drying, and Plant Formation. *In Plant Cell, Tissue and Organ Culture* (pp. 103–113). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-79048-5_9

Atwood, J. T., Dalstrom, S., & Orchidaceae, R. (2002a). *Phragmipedium kovachi*, a new species from Peru. *Selbyana*, *23*, 1–4. https://www.jstor.org/stable/41760112

Atwood, J. T., Dalstrom, S., FERNANDEZ Curador de Orchidaceae, R., & San Marcos, H. (2002b). *Phragmipedium kovachi*, a new species from Peru. In *Selbyana* (Vol. *23*).

Billard, C. E., Barsanti, M. V., & Lallana, V. H. (2015). Cultivo *in vitro* y aclimatación de plantas de Polystachya concreta (Orchidaceae). *FABICIB*, *18*, 95–106. https://doi.org/10.14409/fabicib.v18i0.4847

Bonilla Morales, M. M. (2015). Conservación *in vitro*: Una perspectiva para el manejo de los recursos fitogenéticos. *Revista de Investigación Agraria y Ambiental*, *6*(1), 67–82. https://doi.org/10.22490/21456453.1264

Botelho, F. B. S., Rodrigues, C. S., & Bruzi, A. T. (2015). Ornamental Plant Breeding. *Ornamental Horticulture*, *21*(1). https://doi.org/10.14295/rbho.v21i1.770

Braem, G. J. (2004). Phragmipedium kovachii, Schluckebieria-nouvelle section du genre *Phragmipedium*-et réflexions sur les pratiques *Phragmipedium*. *Richardiana*, *4*(3), 89–102. https://www.researchgate.net/publication/266203885

Cavalcante, M. C., Oliveira, F. F., Maués, M. M., & Freitas, B. M. (2012). Pollinators under Stress: Taxonomy, Physiology, and Behavior of Insect Pollinators. *Psyche: A Journal of Entomology*, *2012*, 978019. https://doi.org/10.1155/2012/978019

CITES. (2021). *Convención sobre el Comercio Internacional de Especies Amenazadas de Fauna y Flora Silvestres* (*CITES*). Apéndices I, II y III. In CITES. https://cites.org/esp/app/index.php

Cortes, A., Pérez Mg, C., & Rm, S. M. (2017). Importancia de los cultivos vegetales Invitro para establecer bancos de germoplasma y su uso en investigación. *Biociencias*, *1*. 71-83. https://dialnet.unirioja.es/servlet/articulo?codigo=9210444

Cozzolino, S., & Widmer, A. (2005). Orchid diversity: An evolutionary consequence of deception? *Trends in Ecology & Evolution*, *20*(9), 487–494. https://doi.org/10.1016/j.tree.2005.06.004

Craig, P. (2005). *The Case of the Purloined Orchid _ Sarasota Magazine*.

Cribb, P. (2005). 511. *Phragmipedium Kovachii*. *Curtis Botanical Magazine*, *22*(1), 8–11. https://doi.org/10.1111/j.1355-4905.2005.00454.x

Cuoco, L. B., & Cronan, J. B. (2009). Orchidaceae: Using a Globalized Commodity to Promote Conservation and Sustainable Economic Development in Southern Ecuador. *Journal of Sustainable Forestry*, *28*(6–7), 799–824. https://doi.org/10.1080/10549810902936623

Davenport, T. R. B., & Ndangalasi, H. J. (2003). An escalating trade in orchid tubers across Tanzania's Southern Highlands: assessment, dynamics and conservation implications. *Oryx*, *37*(1), 55–61. https://doi.org/10.1017/s0030605303000127

David, H., Damián, R., Juan, A., & César, M. (2012). Caracterización morfológica de plantas mediante procesamiento digital de imágenes. *XIII Argentine Symposium on Technology*, 264–275. https://sedici.unlp.edu.ar/handle/10915/123946

De Investigaciones, V., & Extensión, I. y. (2015). *Apropiación social del conocimiento a través de la investigación en la Universidad Tecnológica de Pereira*. Pereira: Universidad Tecnológica de Pereira. https://doi.org/10.22517/9789587222296

Develey, P. F. (2021). Bird Conservation in Brazil: Challenges and practical solutions for a key megadiverse country. *Perspectives in Ecology and Conservation*, *19*(2), 171–178. https://doi.org/10.1016/j.pecon.2021.02.005

Díaz-Toribio, M. H. (2013). *Manual de Cultivo de Orquídeas*. SEV. ISBN-10: 6077579254.

FAO. (2012). *Material de propagación de calidad declarada: Protocolos y normas para cultivos propagados vegetativamente*. https://openknowledge.fao.org/items/75c22232-e66a-4179-ac83-7ac9c10f2922

Faisal, M., & Alatar Abdulrahman, A. (Eds.). (2019). *Synthetic Seeds* (1st Ed.). Springer Cham. https://doi.org/doi.org/10.1007/978-3-030-24631-0

Fatahi, M., Vafaee, Y., Nazari, F., & Tahir, N. A. (2022). *In vitro* asymbiotic seed germination, protocorm formation, and plantlet development of Orchis simia Lam.: A threatened terrestrial orchid species. *South African Journal of Botany*, *151*, 156–165. https://doi.org/10.1016/j.sajb.2022.09.035

Fehér, A. (2019). Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? *Frontiers in Plant Science*, *10*, 536. https://doi.org/10.3389/fpls.2019.00536

Feng, J.-H., & Chen, J.-T. (2014). A Novel *in vitro* Protocol for Inducing Direct Somatic Embryogenesis in Phalaenopsis aphrodite without Taking Explants. *The Scientific World Journal*, *2014*, 263642. https://doi.org/10.1155/2014/263642

Flores-Escobar, G., Legaria-Solano, J. P., Gil-Vásquez, I., & Colinas-León, Ma. T. (2008). PROPAGACIÓN *in vitro* DE Oncidium stramineum Lindl. Una Orquídea Amenazada Y Endémica De México. *Revista Chapingo Serie Horticultura*, *14*(3), 347–353. https://doi.org/10.5154/r.rchsh.2007.02.009

Freudenstein, J. V., & Rasmussen, F. N. (1996). Pollinium Development and Number in the Orchidaceae. *American Journal of Botany*, *83*(7), 813–824. https://doi.org/10.2307/2446258

Freudenstein, J. V., Harris, E. M., & Rasmussen, F. N. (2002). The evolution of anther morphology in orchids: incumbent anthers, superposed pollinia, and the vandoid complex. *American Journal of Botany*, *89*(11), 1747–1755. https://doi.org/10.3732/ajb.89.11.1747

Gantait, S., Kundu, S., Yeasmin, L., & Ali, Md. N. (2017a). Impact of differential levels of sodium alginate, calcium chloride and basal media on germination frequency of genetically true artificial seeds of *Rauvolfia serpentina* (L.) Benth. ex Kurz. *Journal of Applied Research on Medicinal and Aromatic Plants*, *4*, 75–81. https://doi.org/10.1016/j.jarmap.2017.01.005

Gantait, S., Vijayan, J., & Majee, A. (2017b). Artificial Seed Production of Tylophora indica for Interim Storing and Swapping of Germplasm. *Horticultural Plant Journal*, *3*(1), 41–46. https://doi.org/10.1016/j.hpj.2017.06.004

He, L.-F., Qiang, S.-J., & Zhang, Y.-H. (2021). The complete chloroplast genome of Pleione maculata, an orchid with important ornamental value and medicinal value. *Mitochondrial DNA Part B*, *6*(8), 2263–2265. https://doi.org/10.1080/23802359.2021.1948366

Hinsley, A., Verissimo, D., & Roberts, D. L. (2015). Heterogeneity in consumer preferences for orchids in international trade and the potential for the use of market research methods to study demand for wildlife. *Biological Conservation*, *190*, 80–86. https://doi.org/10.1016/j.biocon.2015.05.010

Hossain, M. M. (2015). Ex Vitro Seedling Development from *in vitro* Rhizome-Like Bodies in Eulophia promensis Lindl: A New Technique for Orchid Propagation. *Journal of Botany*, *2015*, 207694. https://doi.org/10.1155/2015/207694

Lee-Espinosa, H. E., Laguna-Cerda, A., Murguía-González, J., Iglesias-Andreu, L., García-Rosas, B., Escobedo-López, D., Martínez-Ocampo, Y. M., Barredo-Pool, F. A., & Santana-Buzzy, N. (2010). Un Protocolo De Embriogénesis Somática Para La Regeneración Y Caracterización *in vitro* De Laelia anceps ssp. dawsonii. *Revista Fitotecnia Mexicana*, *33*(4), 323–332. https://doi.org/10.35196/rfm.2010.4.323

Lee-Espinosa, H. E., Universidad Veracruzana. Facultad de Ciencias Biológicas-Agropecuarias, Laboratorio de Micropropagación Vegetal. Carretera Peñuela-Amatlán,km 1, Peñuela, municipio de Amatlán de los Reyes, Veracruz. México, Murguía-González, J., Laguna-Cerda, A., García-Rosas, B., Gámez-Pastrana, M. R., Galindo-Tovar, M. E., Landero-Tórres, I., Iglesias-Andreu, L., & Santana-Buzzy, N. (2009). Somatic embryoids encapsulation of *Laelia anceps* ssp. Dawsonii to produce synthetic seed. *Revista Chapingo Serie Horticultura*, *15*(2), 33–40. https://doi.org/10.5154/r.rchsh.2009.15.047

Levitus, G., Echenique, V., Rubinstein, C., Hopp, E., & Mroginski, L. (2010). *Biotecnología y Mejoramiento Vegetal II*. ArgenBio.

Li, X., Ding, X., Chu, B., Zhou, Q., Ding, G., & Gu, S. (2008). Genetic diversity analysis and conservation of the endangered Chinese endemic herb Dendrobium officinale Kimura et Migo (Orchidaceae) based on AFLP. *Genetica*, *133*(2), 159–166. https://doi.org/10.1007/s10709-007-9196-8

Chandrasekhara, M. R. (2012). Synthetic seeds: A review in agriculture and forestry. *African Journal of Biotechnology*, *11*(78), 14254–14275. https://doi.org/10.5897/ajb12.770

Mállap-Detquizán, G., Meléndez-Mori, J. B., Huaman-Huaman, E., Vilca-Valqui, N. C., & Oliva, M. (2022). Anther culture in Physalis peruviana L. Microspore stages, sterilization methods and culture media. *Bioagro*, *35*(1), 33–42. https://doi.org/10.51372/bioagro351.4

Martel, C., Egoavil, L., Ocupa, L., Tello, J. A., Rizo Patrón, F., Laura, C., León, M., & Collantes, B. (2021). An homage to Peru's bicentenary: Maxillaria bicentenaria (Orchidaceae), a new species previously misidentified as M. pyhalae. *Phytotaxa*, *518*(2), 87–99. https://doi.org/10.11646/phytotaxa.518.2.1

Menchaca García, R. A. (2011). *Manual para la propagación de orquídeas*. Comisión Nacional Forestal-Mexico.

MINAM. (2006). *Cuarto Informe Nacional Sobre La Aplicación Del Convenio De Diversidad Biologica Años Capítulos I, II, III, IV Apéndices, y Anexo Áreas Protegidas Dirección General de Diversidad Biológica*.

MINAM. (2015). *GUÍA DE Identificación De Orquídeas Con Mayor Demanda Comercial*. MINAM. ISBN-10: 978-612-4174-19-3.

Mirenda, T. (2011). Botanic gardens, education, and orchid conservation strategies: the need for a coordinated approach. *Lankesteriana*, *11*(3), 301–305. https://doi.org/10.15517/lank.v11i3.18285

Nongdam, P., & Tikendra, L. (2014). Establishment of an Efficient *in vitro* Regeneration Protocol for Rapid and Mass Propagation of Dendrobium chrysotoxum Lindl. Using Seed Culture. *The Scientific World Journal*, *2014*, 740150. https://doi.org/10.1155/2014/740150

Oliva-Cruz, M., Goñas, M., García, L. M., Rabanal-Oyarse, R., Alvarado-Chuqui, C., Escobedo-Ocampo, P., & Maicelo-Quintana, J. L. (2021). Phenotypic Characterization of Fine-Aroma Cocoa from Northeastern Peru. *International Journal of Agronomy*, *2021*, 2909909. https://doi.org/10.1155/2021/2909909

Orlando S., G. P., Juan José, Silva Pupo, & Ángel, E. R. (2004). La semilla artificial. Una solución en la biodiversidad mundial. *Cuadernos de Biodiversidad*, *15*, 17–22. https://doi.org/10.14198/cdbio.2004.15.03

Pérez Martínez, B. A. (2015). Establecimiento *in vitro* de Hypericum goyanesii Cuatrec. E Hypericum juniperinum Kunth, a partir del cultivo de semillas. *Revista Colombiana de Biotecnología*, *17*(2), 156–160. https://doi.org/10.15446/rev.colomb.biote.v17n2.54294

Phelps, J., & Webb, E. L. (2015). "Invisible" wildlife trades: Southeast Asia's undocumented illegal trade in wild ornamental plants. *Biological Conservation*, *186*, 296–305. https://doi.org/10.1016/j.biocon.2015.03.030

Prenner, G., Bateman, R. M., & Rudall, P. J. (2010). Floral formulae updated for routine inclusion in formal taxonomic descriptions. *Taxon*, *59*(1), 241–250. https://doi.org/10.1002/tax.591022

Qahtan, A. A., Abdel-Salam, E. M., Alatar, A. A., Wang, Q.-C., & Faisal, M. (2019). An Introduction to Synthetic Seeds: Production, Techniques, and Applications. In M. Faisal & A. A. Alatar (Eds.), *Synthetic Seeds* (pp. 1–20). Springer, Cham. https://doi.org/10.1007/978-3-030-24631-0_1

Rasmussen, H. N., Dixon, K. W., Jersáková, J., & Těšitelová, T. (2015). Germination and seedling establishment in orchids: a complex of requirements. *Annals of Botany*, *116*(3), 391–402. https://doi.org/10.1093/aob/mcv087

Rihan, H., Kareem, F., El-Mahrouk, M., & Fuller, M. (2017). Artificial Seeds (Principle, Aspects and Applications). *Agronomy*, *7*(4), 71. https://doi.org/10.3390/agronomy7040071

Rivera, R. A., & Honduras, D. (2002). *Guía ilustrada de 55 especies de Orquídeas encontradas en la Reserva Biológica de Yuscarán*. Doctoral dissertation, Zamorano: Escuela Agrícola Panamericana.

Saiprasad, G. V. S. (2001). Artificial Seeds and their Applications. *Resonance*, *6*(5), 39–47. https://doi.org/10.1007/BF02839082

Scriven, L. A., Sweet, M. J., & Port, G. R. (2013). Flower Density Is More Important Than Habitat Type for Increasing Flower Visiting Insect Diversity. *International Journal of Ecology*, *2013*, 237457. https://doi.org/10.1155/2013/237457

Segretín, M. E. (1992). *Los cultivos celulares y sus aplicaciones I (cultivos de células animales)*. https://argenbio.org/

Singer, R. B. (2009). Morfología Floral Y Polinización De Orquídeas. *El Segundo Libro De Charles Darwin Floral Morphology and Pollination in Orchidaceae: Charles Darwin's Second Book. In Acta Biol. Colomb*, *14*, 337–348.

Tejada-Alvarado, J. J., Meléndez-Mori, J. B., Ayala-Tocto, R. Y., Goñas, M., & Oliva, M. (2023). Influence of Silver Nanoparticles on Photosynthetic Pigment Content and Mineral Uptake in Pineapple Seedlings Grown *in vitro* under Aluminum Stress. *Agronomy*, *13*(5), 1186. https://doi.org/10.3390/agronomy13051186

T-Thienprasert, N. P., T-Thienprasert, J., Ruangtong, J., Jaithon, T., Srifah Huehne, P., & Piasai, O. (2021). Large Scale Synthesis of Green Synthesized Zinc Oxide Nanoparticles from Banana Peel Extracts and Their Inhibitory Effects against *Colletotrichum* sp., Isolate KUFC 021, Causal Agent of Anthracnose on Dendrobium Orchid. *Journal of Nanomaterials*, *2021*, 5625199. https://doi.org/10.1155/2021/5625199

Utami, E. S. W., & Hariyanto, S. (2019). *In vitro* Seed Germination and Seedling Development of a Rare Indonesian Native Orchid Phalaenopsis amboinensis JJSm. *Scientifica*, *2019*, 8105138. https://doi.org/10.1155/2019/8105138

Vaca, I., Marulanda, M., Verdesoto, J., Núñez, A., Acurio, R. D., & Chiluisa-Utreras, V. (2018). Efecto del carbón activado en la germinación y brotación *in vitro* de *Citrus limon* (L.) y su dinámica de crecimiento. *Bionatura*, *3*(3), 657–664. https://doi.org/10.21931/rb/2018.03.03.5

Vermeulen, P. (1966). The system of the Orchidales. *Acta Botanica Neerlandica*, *15*(1), 224–253. https://doi.org/10.1111/j.1438-8677.1966.tb00228.x

Vohra, P., & Gahlawat, I. N. (2020). Anthropogenic activities led impact on Biodiversity and need of proactive endeavours towards sustainable ecosystems through life diversity conservation. *Integrated Journal of Social Sciences*, *7*(2), 31–42. http://www.pubs.iscience.in/journal/index.php/ijss/article/view/1123

William, N. H. (2013). *Manual de orquídeas*. https://repositoriodigital.minam.gob.pe/handle/123456789/880